X-641-71-163
PREPRINT

AN AUTOMATED LUCIFERASE ASSAY OF BACTERIA IN URINE

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APRIL 1971

GSFC
GODDARD SPACE FLIGHT CENTER
GREENBELT, MARYLAND

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AN AUTOMATED LUCIFERASE ASSAY OF BACTERIA IN URINE

INTRODUCTION

The many advances in scientific and engineering knowledge produced by NASA space research often apply to major problems of modern society. To assist in the application of new technology and thus reduce the time interval from space development to direct benefit to society, NASA has established a technology-application effort at Goddard Space Flight Center (GSFC). As part of this effort, GSFC is using technology for the detection of extraterrestrial life to develop a simple, fast, and accurate procedure for detecting and counting bacteria in urine. In the United States, where the incidence of urinary-tract infection is second only to that of respiratory-system infection[1], the examination of urine for bacteria is one of the most frequent and important clincial assays.

In the routinely used culture-colony count, a urine specimen is streaked on nutrient agar plates and allowed to incubate so that any bacteria present will reproduce. The resulting colonies are counted and this count is compared with standard guide numbers to determine if the specimen contains infection.* The cultive-colony count has two disadvantages. The first is that a low count may be is misleading: a serious infection may consist of bacteria which are unable to grow and reproduce in a culture medium. The second is that the requirement for the bacteria to incubate causes a delay in diagnosis of 12 to 96 hours.

A direct cell count avoids these disadvantages, but has other limitations. Of the existing direct cell count procedures, the most well-known are done with the microscopic counting chamber[2] and the Coulter apparatus[3]. Microscopic counting of bacteria is too tedious and time-consuming to be done routinely in a large clinical laboratory. The Coulter apparatus, sensitive to inert particulate matter as well as organisms, does not attain accurate results when urine specimens contain these inert particulates. Another methods for a direct cell count depends on the ability of organisms to reduce nitrates to nitries. This method, while simple, has failed to detect 20 to 30 percent of urinary-tract infections[4].

*According to accepted standards, a final count of less than 10,000 bacteria cells per ml of urine indicates that the bacteria are from sources outside the urinary tract: between 10,000 and 100,000 cells per ml, a probable infection: and over 100,000 cells per ml, an infection.

BIOLUMINESCENCE ASSAY FOR ATP

The GSFC procedure, a new method for a direct cell count, as based on the assay of adenosine triphosphate (ATP) in urine specimens. ATP is a constituent of all living cells[5] and hence, of bacteria. Tests on pure cultures of several bacteria species showed that the concentration of cellular ATP can be correlated with the number of bacteria cells present[6].

The most sensitive method now available for measuring ATP is based on the bioluminescent reaction of luciferase when mixed with ATP, the reaction which also creates the glow in the tail of a firefly. McElroy first demonstrated that ATP is required for this reaction; Strehler further investigated this reaction to develop a highly sensitive method for measuring ATP.

The reaction occurs in two steps:[7]

$$E + LH_2 + ATP \xrightleftharpoons{Mg^{++}} E \cdot LH_2 \cdot AMP + PP$$

$$E \cdot LH_2 \cdot AMP + O_2 \longrightarrow E + AMP + CO_2 + h_\nu + T$$

where

E = firefly luciferase
LH_2 = reduced luciferin
ATP = adenosine triphosphate
AMP = adenosine monophosphate
PP = pyrophosphate
T = thiazolinone (dehydroluciferin)
h_ν = light (550 mμ)

The maximum intensity of emitted light in this reaction has a direct linear relationship to the concentration of ATP added. This relationship exists over a range determined by a ratio of the concentration of luciferin-luciferase to that of ATP and by the sensitivity of the light-measuring instruments. When purified preparations of luciferase and luciferin are used with a sensitive light-measuring instrument, as small a quantity of ATP as 10^{-5} μgm can be measured accurately (Figure 1).

Procedure Using Luciferase to Measure Bacteria in Urine

The firefly luciferase assay for ATP was adapted to the detection and counting of bacteria in urine specimens. During testing of the procedure, a photometer was used to measure the light reaction.

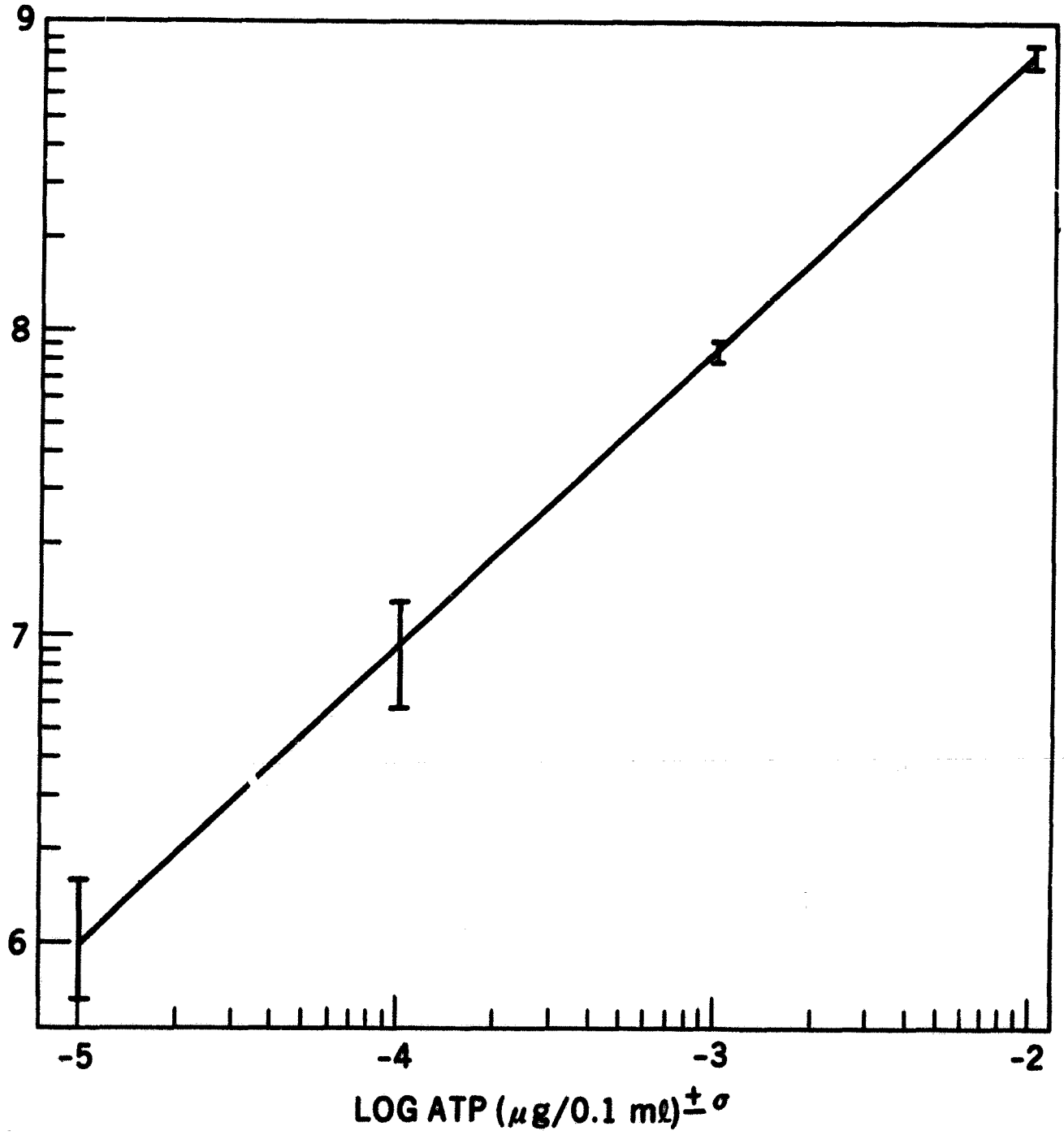

Figure 1. Linear Regression of Initial Light-Peak Height versus ATP Concentration

Figure 2 is a schematic drawing of the light-measuring instrumentation used in the manual ATP assay. It consists of a reaction chamber, photomultiplier assembly, amplifier, power supply, and recorder.

The reaction chamber, coupled to a photomultiplier, is a rotary drum. It is designed so that no light can enter the chamber when it is rotated to permit a luciferase injection into a vial in front of the photomultiplier.

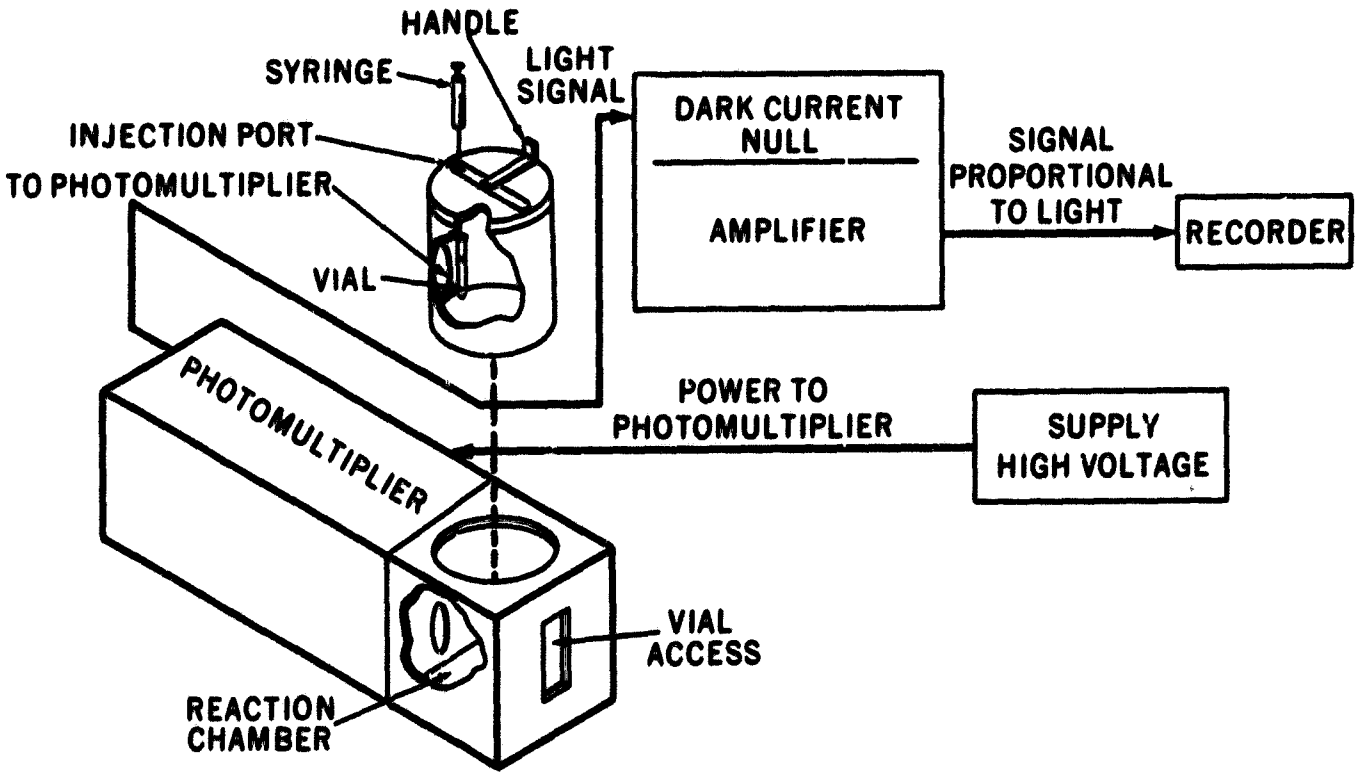

Figure 2. Instrument Used in the Manual ATP Assay

Over 400 urine specimens were tested using the following procedure:

a. To allow for detection of only bacterial ATP in the urine specimens, nonbacterial ATP has to be removed. One ml of urine was mixed with 0.1 ml of 1 percent Triton X-100* to lyse leukocytes, erythrocytes, and tissue cells, and 0.1 ml of the solution of apyrase (an ATPase) to hydrolyze this released ATP and any soluble ATP excreted in the urine. This mixture was allowed to stand at room temperature for 10 minutes.

b. The mixture was placed in a 95 to 98°C water bath for 10 minutes to destroy the action of the apyrase.

c. After the mixture was cooled to room temperature, it was combined with 0.1 ml of 1 N perchloric acid and allowed to stand for 5 minutes.

d. This mixture was then neutralized by the addition of 0.1 ml of 1 N KOH, and brought to a final pH of 7.4 by the addition of 0.1 ml of 2 M TES buffer.

*Rohn and Haas TM for octyl phenoxy polyethoxyethanol

e. One tenth ml of this mixture was injected by needle and syringe into a vial containing 0.3 ml of the luciferin-luciferase mixture (Figure 2).

To determine the maximum intensity of light given by a particular concentration of ATP, known concentrations of ATP were substituted for the urine sample, the luciferase mixture added, and the emitted light measured. Figure 3 is a curve showing the sudden light increase that occurs when ATP is added to luciferase solution. Specimens containing bacterial ATP emitted a light with a maximum intensity proportional to the concentration of ATP and hence, of bacteria.

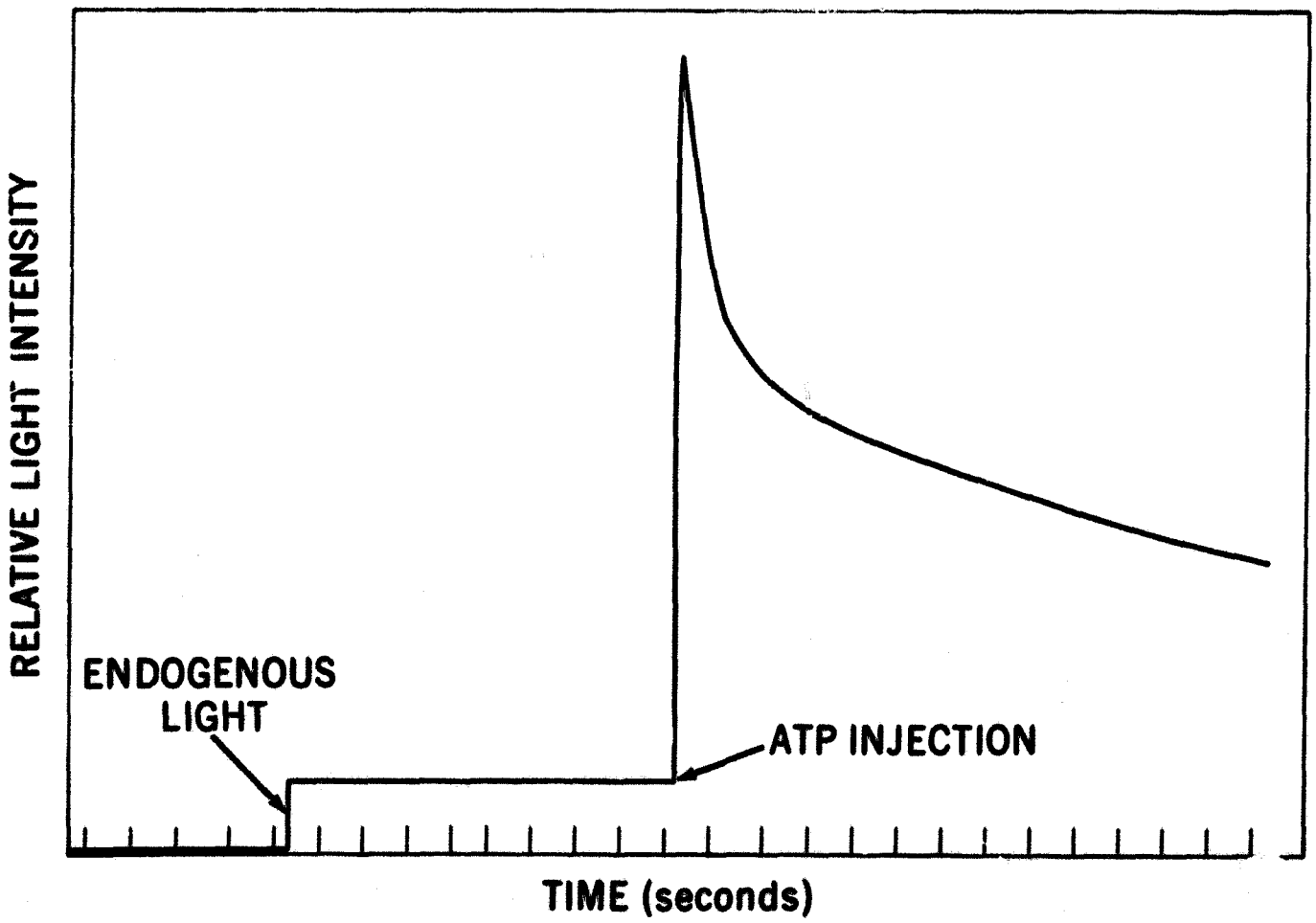

Figure 3. Curve Showing Light Increase When ATP is Added to a Luciferase Solution

Microbiological Methods of Examining Urine for Bacteria

- Culture-colony method: A 0.01 ml loop was used to streak urine samples on two plates: one containing 5 percent blood agar; the other, desoxycholate agar. The plates were incubated for 24 hours at 35°C, then examined. Bacteria colonies were counted.

- Culture of pure bacteria strains: Pure species were removed from standard urine culture plates and transferred to an infusion broth. After an incubation period which lasted well into the logarithmic growth phase, aliquots of the cells were removed for pour-plate counting and an ATP assay.

- Viable cell count by pour plate: Urine samples were serially diluted in isotonic saline. One-tenth ml aliquots of each dilution were placed in sterile petri dishes, covered with 15 ml of trypticase soy agar, and swirled to ensure adequate mixing. The plates were incubated overnight at 35°C, and the colonies were counted the following morning.

- Total cell count: A Petroff-Hausser counting chamber was used for a direct microscopic count of the total number of bacteria in urine samples treated with 0.1 percent Triton X-100.

Comparison of the ATP Assay to Other Methods of Urine Examination

When a colony count showed that specimens had 10,000 colonies per ml or more, an ATP assay of the same specimens showed positive results. About 20 percent of the urine specimens with a negative culture-colony count showed positive ATP results (Table 1).

To assess how results from the ATP assay correlated to results from a culture-colony count, the average ATP content of a bacteria cell was determined. Although Chappelle had previously found 2×10^{-10} μgm of ATP per cell[6], the possibility existed that this value might change when the bacteria were measured in urine. In an ATP assay of the following nine bacteria species commonly found in urine, the ATP content per cell ranged from 0.5 to 8.2×10^{-10} μgm.

Species	μgm ATP per cell ($\times 10^{-10}$)
Escherichia coli	1.2
Streptococcus faecalis	1.1
Staphylococcus aureus	1.8
Staphylococcus epidermidis	8.2
Proteus mirabilis	4.3
Klebsiella	2.8
Pseudomonas	0.5
Proteus rettgeri	1.4
β streptococcus	1.5

The value used for this conversion of ATP concentration into cells per ml was 3×10^{-10} μ gm.

Results from selected urine specimens were used to determine a relationship between the number of bacteria found by culture-colony count and the number of bacteria calculated from the ATP response. The ATP assay revealed bacteria in so many more specimens than the culture-colony count did that results from the culture-colony count were compared to results from the more accurate pour plate technique (Table 2). Although the pour plate procedure revealed more bacteria cells than the culture-colony count method did, the results were still much lower than those from ATP measurements or microscopic count (Table 3).

Table 1

Comparison Between Culture-Colony Counting and ATP Assay

Number of speimens tested	282	78	39	57
Number of colonies counted	0	1 to 10,000	10,000 to 100,000	Over 100,000
Number of positive ATP responses	60	42	39	57

Table 2

Number of Bacter per ml Urine Sample Determined by ATP Assay, Culture-Colony Plate, and Pour Plate

Sample Number	ATP Method	Culture-Colony Plate	Pour Plate
1	7×10^8	0	1×10^4
2	4×10^7	1×10^4	1×10^7
3	3×10^7	1×10^4	2×10^7
4	3×10^7	0	0
5	5×10^9	2×10^4	3×10^5
6	3×10^7	0	0
7	8×10^7	0	0

Table 2 (Continued)

Number of Bacter per ml Urine Sample Determined by ATP Assay, Culture-Colony Plate, and Pour Plate

Sample Number	ATP Method	Culture-Colony Plate	Pour Plate
8	6×10^7	1×10^5	2×10^7
9	2×10^8	1×10^4	8×10^4
10	4×10^7	1×10^3	0
11	5×10^8	1×10^5	3×10^7
12	3×10^7	0	0
13	1×10^7	0	2×10^3
14	8×10^7	0	4×10^3
15	8×10^7	4×10^4	9×10^3
16	7×10^9	1×10^5	2×10^8
17	8×10^7	1×10^5	5×10^6
18	2×10^7	2×10^3	5×10^3
19	9×10^9	1×10^5	2×10^7
20	4×10^8	1×10^4	3×10^5
21	2×10^8	1×10^4	4×10^6

In tests on the various cell-count methods, results from the ATP assay correlated closer to results from direct cell count with a microscope than did results from any other method. These tests demonstrated the validity of the ATP assay as a direct cell count procedure. However, a cell count by ATP assay was often much higher than a corresponding culture-colony count. Even when the culture-colony count was less than 1000 cells, the cell count by ATP assay was often about 10 million cells, this number being confirmed by cell count with a microscope (Table 3). The total significance of this discrepancy has not yet been determined.

Table 3

Number of Bacteria per ml of Urine Sample Determined by ATP Assay, Pour Plate, and Microscope Count

Sample	ATP Method	Pour Plate	Microscopic
1	8×10^7	4×10^3	6×10^7
2	8×10^7	9×10^3	7×10^8
3	7×10^9	2×10^8	2×10^9
4	9×10^9	2×10^7	2×10^8
5	4×10^8	3×10^5	8×10^7
6	7×10^8	1×10^4	2×10^7
7	4×10^7	1×10^7	2×10^8
8	3×10^7	2×10^7	4×10^8
9	5×10^9	3×10^5	1×10^8
10	3×10^7	0	2×10^7
11	8×10^7	0	2×10^7
12	6×10^7	2×10^7	3×10^8
13	2×10^8	8×10^4	2×10^7
14	4×10^7	0	4×10^7
15	5×10^8	3×10^7	7×10^8
16	3×10^7	0	2×10^7
17	1×10^7	2×10^3	1×10^9

Several circumstances may prevent bacteria cells from growing and reproducing:

- An infection may consist mostly of anaerobic bacteria which cannot grow under standard culture conditions. Anaerobic culture tests made on urine specimens revealed large numbers of anaerobic organisms in many specimens. These numbers correlated well with results from both the microscopic and the ATP counts.
- Aerobic cells occurring in a chronic urinary-tract infection may be unable to grow and divide in the temperature, pH, gas phase, and nutrient medium used in the colony-count method.

- The bacteria cells in a urine specimen might be metabolically dead. This implies than an accumulation of dead bacteria cells exists from a chronic infection, or that the cells from a recent infection have been killed.

- The bacteria cells may be viable, but a high concentration of antibiotic in the urine specimen may prevent them from dividing.

Because the ATP assay detects cells that the standard urine-examination techniques miss, the scope of usefulness of the ATP assay may be much broader. To determine this, follow-up experiments are planned. Patients with symptons of urinary-tract infection will be tested subsequently by both the standard techniques and the ATP assay. Results of the tests will be compared with the frequency that urinary-tract infection recurs in those patients.

AUTOMATION OF THE PROCEDURE

GSFC has adapted the ATP assay to a prototype instrument which performs the ATP assay in 15 minutes. This automated instrument, (Figure 4), called the FLASH for "fast luciferase automated assay of specimens for hospitals," reads out results for a specimen every minute.

It is expected that when the FLASH is used in a large hospital laboratory, about half of all specimens processed will show a negative ATP response. These specimens will require no further processing. Specimens containing ATP may be further analyzed by routine hospital procedures to identify the bacteria and to determine which antibiotics will be used in treatment.

The FLASH has two major components: a reaction unit (Figure 5) where the chemical process takes place, and a pump unit (Figure 6) which dispenses reagents into the reaction unit. A recorder is used for display of results.

In the FLASH, each specimen is processed in a separate vial. The vials, set in a turning table, (Figure 5) strike a series of switches suspended from the table cover. These switches activate pumps to inject reagents into the urine.

After the luciferase mixture is injected into a specimen, the vial passes in front of a photomultiplier-amplifier system which detects any light emitted and converts this light into a signal to a strip-chart recorder. The readout is a peak showing the amount of light emitted from the specimen, the height of the peak correlating to the amount of ATP and thus to the amount of bacteria in each specimen[6].

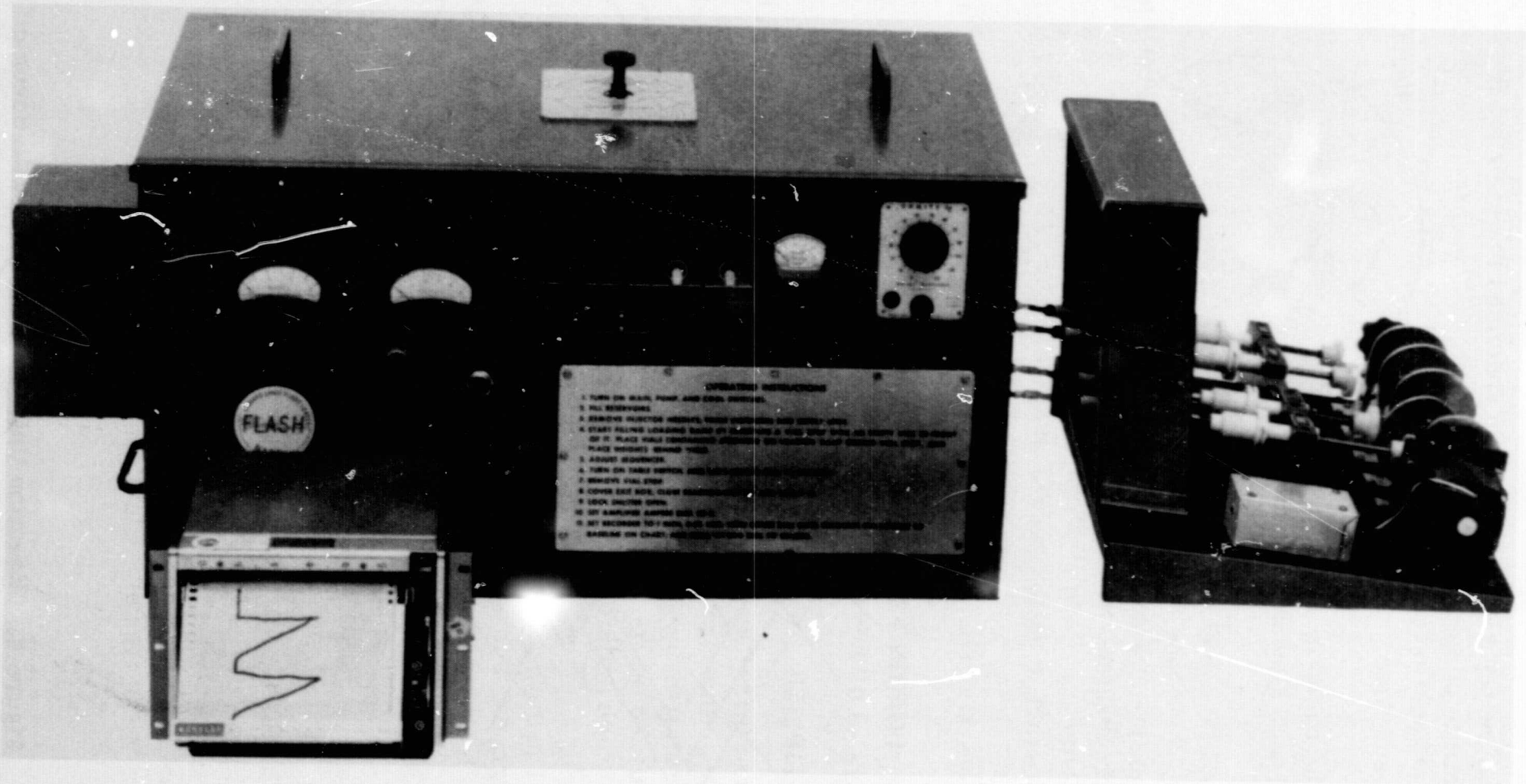

Figure 4. The FLASH

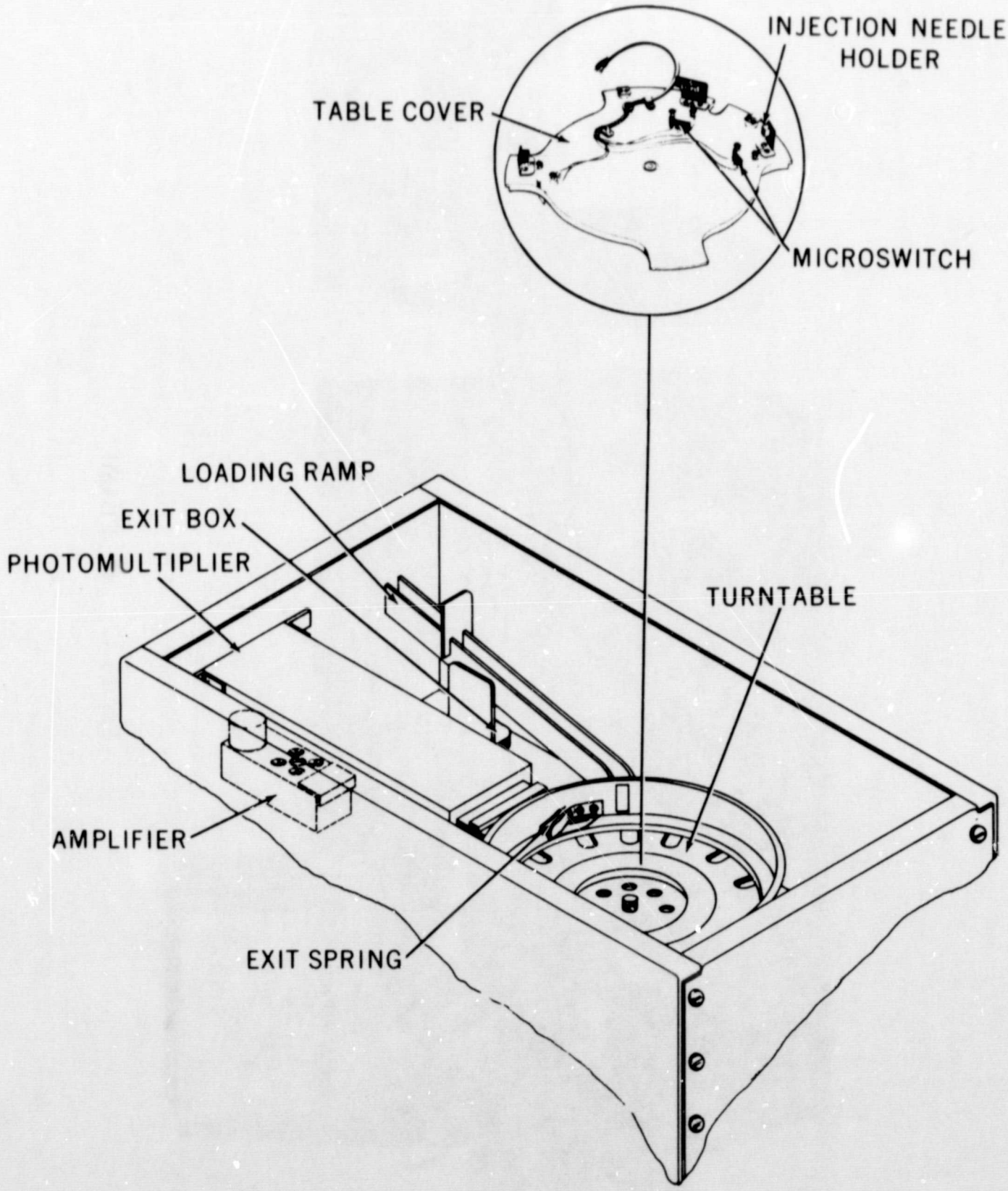

Figure 5. Reaction Unit (showing table with cover off)

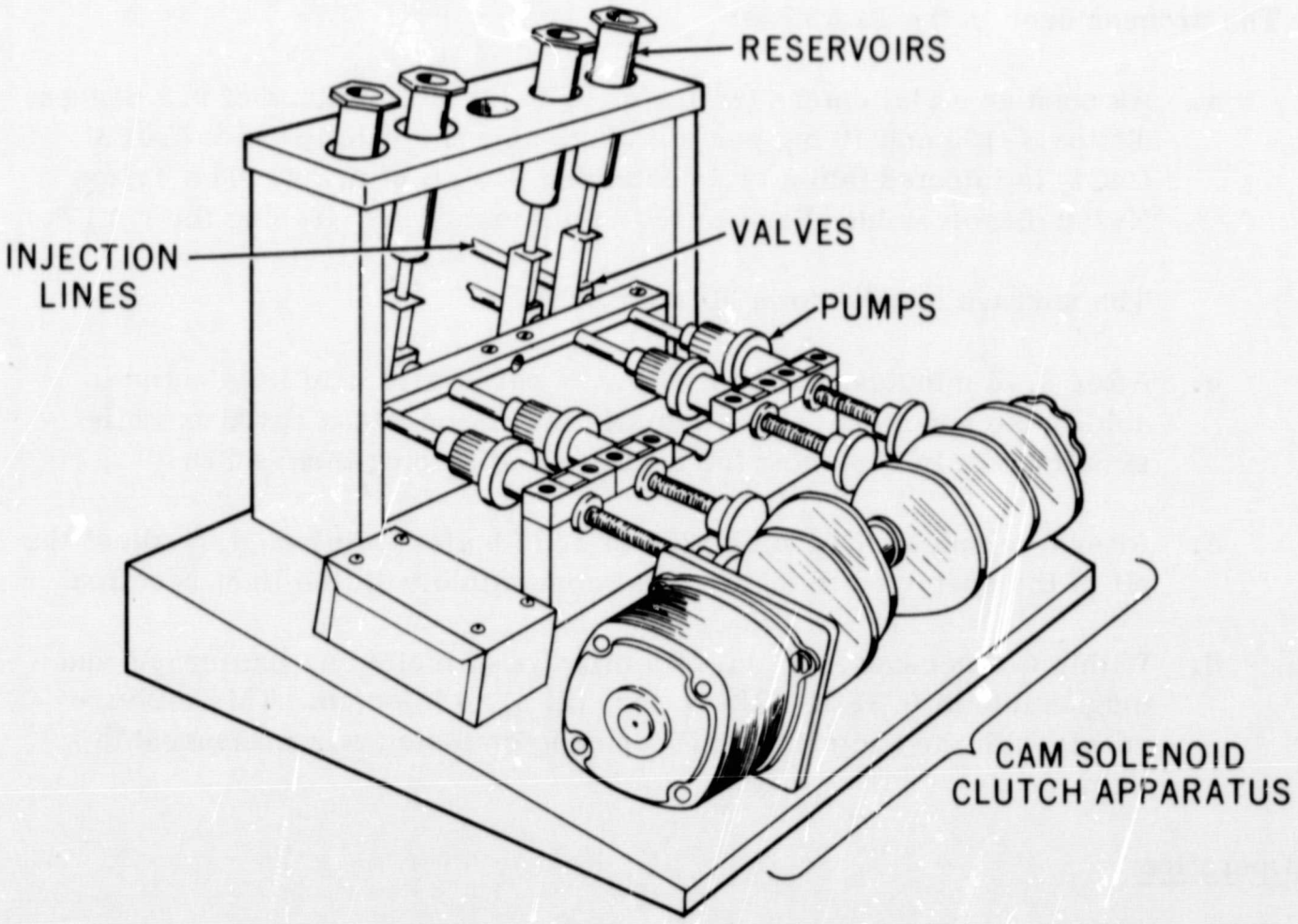

Figure 6. Pump Unit

To make the ATP assay more compatible with a compact instrument, the volumes of the chemicals used were scaled down. To simplify the process, the heat step was eliminated. It was found that perchloric acid functions equally well in stopping the apyrase action before this action destroys the bacterial ATP.

The KOH, used to neutralize the perchloric acid, thereby reactivated the apyrase and also partially inhibited the light reaction. KOH was eliminated from the process; to neutralize the perchloric acid, a strong high molar buffer is now added immediately before the luciferase.

However, the high concentration of buffer in a specimen disturbs the balance of ionic strengths between the luciferase mixture and the processed urine specimen. To compensate for this, the luciferase solution is made up in 0.31 M TRIS.

The ATP Process Adapted to Automation

The process used in the FLASH is:

a. As soon as a vial enters the table, 0.02 ml of a mixture of 0.5 percent Triton X-100 and 10 mg per ml potato apyrase solution with 0.01 M $CaCl_2$ is injected into a vial containing 0.1 ml of urine. The Triton X-100 dissolves blood and tissue cell membranes, freeing their ATP.

   The apyrase breaks down all free ATP.

b. After 8.75 minutes, 0.02 ml of 0.5 N perchloric acid is injected to acidify the specimen. This inhibits the action of the apyrase while simultaneously rupturing the bacteria and freeing their ATP.

c. After 5 minutes, 0.02 ml of 2.5 M TRIS buffer is injected to adjust the pH of the specimen to 8, the level compatible with the light reaction.

d. Within 0.5 seconds, 0.4 ml of a mixture of luciferin, luciferase, and magnesium in 0.31 M TRIS buffer, pH 8, is injected. This mixture reacts with the bacterial ATP, causing emission of a measureable light.

Operation

The FLASH is easy to operate. An operator turns on the pump unit, fills reservoirs in that unit with the various reagents, primes the pump system, and calibrates the volume of each reagent to be injected by adjusting the pumps. He then loads 30 vials of urine onto the loading ramp, closes the reaction-unit lid to seal out ambient light, turns on the turntable, and opens the shutter protecting the photomultiplier. Processing of the specimens is entirely automatic, so the operator's only remaining duty is to refill the loading ramp every 30 minutes.

System Verification

To verify that the luciferase remains potent, a control solution can be processed after every nine specimens. This solution consists of lyophilized aliquots with known quantities of bacteria normally found in urinary-tract infections such as (Escherichia coli). To verify that the photomultiplier is operating correctly, a radioactive light standard can be inserted in front of the photomultiplier lens.

Construction

Reaction Unit

In the reaction unit (Figure 5), openings spaced evenly around the turntable hold 15 vials at a time. Two flanges inside a cylinder surrounding the table keep the vials in these openings.

A U-shaped loading ramp is held at an angle which permits the vials to gravity-feed onto the table. Two round metal weights can be placed behind the vials to prompt loading. The inside of this ramp is coated with teflon to permit the vials to slide. A U-shaped exit spring pushes the processed vials onto an exit chute into a removable box.

A 100 inch ounce synchronous motor, which turns at 1/3 rpm, is geared to permit the table to complete one revolution every 15 minutes.

The cover position keys the position of the microswitches and injection needles to the proper timing of the process. If the point where the vial enters the table is considered as 0 degrees, the first injection is at 6 degrees, the second at 192 degrees, and the third and fourth at 312 degrees.

Air Circulation. To ensure that the temperature does not exceed optimal for the luciferase, a 4-inch muffin fan is mounted behind the photomultiplier tube, and the rear of the reaction unit is vented. The table cover has cutouts and the outer ring of the table is raised so air can circulate through the table.

Light Sealing. To ensure that even very low levels of ATP can be measured, outside light and internal reflections are totally restricted from the reaction unit. The reaction-unit is covered during operations and a shutter over the photomultiplier lens can be closed to prevent light from striking the photomultiplier when the lid is off. The fan shroud and air vents are both light-baffled.

To prevent light from the processed vials from interfering with the reading for the vial in front of the photomultiplier, the exit chute and box are both covered, and a trap door at the end of the chute swings shut after the vial passes into the box.

Light-Detection Instrumentation. A 14-stage photomultiplier with an S-20 photocathode detects light from the processed vials. The photomultiplier has a dark current of 10^{-11} amperes which is nulled by the amplifier offset. The photomultiplier runs at -1550 vdc, and the amplifier has a sensitivity range of four decades.

Readout

FLASH uses a strip-chart recorder with a half-second full-scale pen response to follow the light output. Another pen on the recorder, activated by the solenoid at the luciferase pump, indicates the injection of the luciferase mixture into each vial.

Pump Unit

In the pump unit (Figure 6), each reagent pump is slanted up toward the reaction unit to eliminate air from the pumping system. The pump motor turns a shaft continuously at 18 rpm. Whenever a vial in the reaction unit strikes a switch on the turntable cover, a signal actuates the solenoid-controlled clutch and cam associated with that switch. The clutch locks onto the pump-motor shaft, turning the cam once to operate the corresponding pump. This pump ejects the reagent through its dispensing valve, tubing, and needle into a specimen vial and, on the return stroke, draws in reagent through its intake valve.

The 20-ml disposable plastic syringes which act as reservoirs may be changed daily. Materials used in the components of the pump unit were chosen for their compatibility to all reagents used. Although other reagents used in the process are injected through disposable needles, the luciferase mixture is injected through a permanent teflon needle.

Construction Materials

Component	Material
Turntable system	aluminum
Turntable cover	acrylic sheet
Reservoirs, valves	polypropylene
Pumps	glass
Valve spring	elgiloy
Ball valve	silicon rubber
Flapper valve	silicon rubber

Potential Applications

The FLASH process and instrument could be adapted to suit other useful applications. The following components of the machine are adjustable:

- Dispensers: The volume of fluid injected into the reaction unit can range from 0.02 ml to 0.45 ml per injection; the number of reagents dispensed can be varied

- Turntable cover: Locations of switches and injection needles can be changed to change the time lag between injections

- Motor: Gearing can be changed or a different motor used to change the total time of the process. This time would be limited only by the amount of time required to make the light reading. Results can be obtained as quickly as one in 0.5 minute or as slowly as one in 4 hours.

The FLASH instrument has the potential to be adapted to:

- Testing for bacterial sensitivity to antibiotics

- Monitoring levels of bacteria built up in industrial products such as cosmetics, drugs, latex paints, and laquers

- Determining bacteria levels in other aqueous body fluids such as lymph, plasma, blood, spinal fluid, saliva, and mucous

- Determining levels of bacteria in the environment

REFERENCES

1. Paul D. Hoeprich. Culture of the Urine. J. Lab. and Clin. Med. LVI, issue 6, 899-907. 1960

2. Graham S. Wilson and A. A. Miles. Topley and Wilson's Principles of Bacteriology and Immunity. 5th ed., I, 101. 1964

3. J. P. Truant, W. A. Brett, and K. E. Merckel. Application of an electronic counter in the evaluation of significant bacteriuria. Henry Ford Hosp. Med. Bulletin. X, 359. 1962

4. L. G. Smith, W. R. Thayer, E. M. Malta, and J. P. Utz. Relationships of the Greiss nitrite test to bacterial culture in the diagnosis of urinary tract infection. Ann. Intern. Med. LIV, 66. 1961

5. F. M. Huennekens and H. R. Whitely. Phosphoric Acid Anhydrides and other Energy-Rich Compounds. Comp. Biochem. I, 107-180. 1960

6. E. W. Chappelle and G. V. Levin. The Use of the Firefly Bioluminescent Assay for the Rapid Detection and Counting of Bacteria. Biochem. Med. II, 41-52. 1968

7. W. D. McElroy, H. H. Seliger, and E. H. White. Mechanism of Bioluminescence, Chemiluminescence, and Enzyme Function in the Oxidation of Firefly Luciferin. Photochem. Photobiol. X, 153-170. 1969

APPENDIX A

Preparation of Reagents for the ATP Assay

Luciferin. Synthetic luciferin[7], a benzothiazole derivative, was prepared with the starting compound 6-ethoxy benzothizaloe-2-sulfonamide.*

Luciferase. One gram of acetone powder prepared from dried firefly lanterns** was extracted with 10 ml of cold 0.05 M N TRIS (hydromethyl) methyl-2-aminoethanesulfonic acid*** i.e., TES buffer, pH 8, containing 0.001 M dithiothreitol (Clelands Reagent) for 30 minutes with stirring. After centrifuging the extract at 13,000 X g for 15 minutes, the supernatant solution was placed on a Sephadex G-200 column (40 x 2.5 cm, 200-ml bed volume) previously equilibrated with the TES buffer. The protein was eluted into 10-ml fractions using a total of 200 ml of the TES. Two-tenths ml aliquots were removed from each fraction and mixed with 0.1 ml of 0.05 M TES buffer containing $MgSO_4$ (0.1 M) and luciferin (0.003 M). To determine the activity of each fraction, the maximum intensity of the resulting light was measured after 0.1 ml of ATP (0.01 nM) was added to the above mixtures. Fractions containing 50 percent of the total activity were pooled and mixed.

Potato Apyrase. Apyrase may be obtained in a partially purified state from Sigma Chemical Company**** or can be easily prepared in the laboratory with a modification of the method described by Traverso-Cori[9].

Five hundred grams of peeled, diced potatoes were added to 250 ml of cold, distilled water and homogenized in a blender for 5 minutes. The suspension was filtered through two layers of cheese cloth and the filtrate taken to 20 percent saturation with $(NH_4)_2SO_4$. The pH was adjusted to 4, and additional $(NH_4)_2SO_4$ was added until the solution reached a 40 percent saturation. The suspension stood 30 minutes at room temperature, and was then centrifuged at 13,000 X g for 15 minutes. The precipitate was discarded, and the supernatant was brought to 70 percent saturation with $(NH_4)_2SO_4$. The suspension stood 30 minutes at room temperature, then was centrifuged at 13,000 X g for 15 minutes. The precipitate was taken up in 50 ml of distilled water.

---

* Upjohn Inc., Kalamazoo, Michigan
** Antonik Laboratories; Elk Grove, Illinois
*** Sigma Chemical Company: St. Louis, Missouri
**** To use the Sigma apyrase preparation, mix it in 0.01 M $CaCl_2$, pH 6.3, in a concentration that will allow 0.1 ml to hydrolyze 1 μgm of ATP in 5 minutes.

The extract was placed on a Sephadex G-100 column equilibrated with 0.01 M $CaCl_2$ in 0.05 M TES buffer, pH 7.4. The column was eluted with the same solution.

To assay the resultant fractions for apyrase activity, 0.1 ml of each fraction was added to 1 μgm of ATP contained in 1 ml of solution. This mixture stood for 5 minutes, then the remaining amount of ATP was measured by the firefly luciferase assay. Those fractions which completely hydrolyzed ATP in 5 minutes were pooled, frozen, and stored for later use.